

Serological Classification by Monoclonal Antibodies of *Rickettsia tsutsugamushi* Isolated in Korea

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Antigenic types of 113 strains of *Rickettsia tsutsugamushi* isolated from Korean patients were analyzed by using murine polyclonal and monoclonal antibodies. The isolates can be classified in six groups according to their reaction to a panel of monoclonal antibodies. Nine isolates of group I were identified as the Gilliam serotype, and 13 isolates of groups II and III were identified as the Karp serotype. There were two groups that were considered to be a mixture of groups I and II or groups I and III, respectively. The remaining 88 strains of group IV had a unique antigenic determinant that was not present in the prototype strains (Karp, Kato, Gilliam), in addition to sharing common antigens with the prototype strains. Therefore these strains, which are more prevalent in Korea, seem to belong to a new serotype closely related to the Karp serotype.

Tsutsugamushi disease in Korean residents was first reported in 1986 and has been found to be prevalent in all areas of Korea (3). *Rickettsia tsutsugamushi*, the causative agent of tsutsugamushi disease, shows multiple serotypes, and this antigenic heterogeneity is of major concern in the serodiagnosis and development of a vaccine for this disease (17). In recent years a number of strains of *R. tsutsugamushi* which are antigenically distinct from the prototype strains (Gilliam, Karp, Kato) have been isolated in Thailand and Japan (5, 16, 18). So it was very interesting to study the antigenicity of the Korean isolates and their antigenic relationship with the prototype strains.

We have previously reported the serotyping of 64 Korean strains of *R. tsutsugamushi* by an immunofluorescent-antibody (IFA) test with mouse hyperimmune sera against prototype strains. But some strains were difficult to classify with hyperimmune sera because of serological cross-reaction (8). To circumvent this difficulty, we produced monoclonal antibodies (MAbs) against the prototype strains of *R. tsutsugamushi*. Recent studies from other groups have shown the usefulness of MAbs for classifying the serotypes of newly isolated strains (4, 7).

We isolated 113 strains of *R. tsutsugamushi* from patients in various areas of Korea and produced several MAbs that reacted to the prototype strains and one of the isolated strains. With these MAbs, we found that most of our isolates were distinct from the prototype strains and that the distribution of the prevalent serotype varied among different areas in Korea.

MATERIALS AND METHODS

Rickettsiae. The prototype strains of *R. tsutsugamushi*, Gilliam (ATCC VR-312), Karp (ATCC VR-150), and Kato (Nigatta strain, kindly provided by H. Tanaka, The Institute of Medical Science, University of Tokyo), were propagated in monolayers of L cells, which were cultured in minimum essential medium containing 2% fetal bovine serum.

To isolate local strains of *R. tsutsugamushi*, blood samples were injected intraperitoneally into three ICR mice.

Fourteen days after injection, the spleens and livers were harvested from the mice and homogenized. The homogenates were used to inoculate L-cell cultures. One hundred and thirteen strains were isolated and cultivated in L cells and used for this study.

Production of MAbs. Female BALB/c mice 5 to 6 weeks old were injected intraperitoneally with a 10% homogenate of the spleens of the BALB/c mice infected with each of the prototype strains or an isolated strain (B92). After 13 to 14 days, hybridomas were prepared by fusing spleen cells and P3x63Ag.653 myeloma cells as previously described (9).

One fusion experiment was performed for each of the four rickettsial strains. Antibodies in the hybridoma culture supernatant were detected by an indirect immunofluorescence test (2), in which L cells infected with rickettsiae were used as antigens, and fluorescein-labeled goat antibodies to mouse immunoglobulin (Cappel Laboratories) were used as the second antibodies. Hybridomas that produced antibodies were cloned by limiting dilution and propagated. Then the hybridoma cells were injected into the peritoneal cavity of the BALB/c mice to produce ascitic fluid as previously described (9). This ascitic fluid containing MAbs to each rickettsial strain was used for the IFA test.

IFA test. Three prototype strains and 113 isolates were grown in L-cell cultures, which were then used as antigens in the IFA test. The infected L cells were smeared onto spot slides and fixed with acetone.

Mouse hyperimmune sera against three prototype strains were prepared as previously described (8). Mouse hyperimmune sera and MAbs, including five clones produced in this study and three clones that were kindly provided by Y. Kobayashi, Faculty of Medicine, Ehime University, Ehime, Japan, were serially diluted from 1:100 to 1:25,600 and reacted with each of the antigen preparations at 37°C for 30 min. After two washes of 5 min each, fluorescein-labeled goat anti-mouse immunoglobulin G (heavy and light chain specific) antiserum was added, and incubation was continued. These stained preparations were mounted with glycerol and examined with a fluorescence microscope. The antibody titers of MAbs are expressed as reciprocals of the highest dilutions that showed visible rickettsial particles.

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TABLE 1. Reaction of MAbs with *R. tsutsugamushi* strains

MAB	Immunogen	IFA titer ^a			
		Gilliam	Karp	Kato	B92
GM165	Gilliam	6,400	— ^b	—	—
GILMA ^c	Gilliam	12,800	—	—	—
KP10	Karp	—	12,800	—	—
KAPMA ^c	Karp	—	6,400	—	—
KP5	Karp	—	12,800	6,400	6,400
KT410	Kato	—	—	12,800	—
KATMA ^c	Kato	—	—	6,400	6,400
KI4	B92	—	—	—	25,600

^a Reciprocal of the highest dilution of ascitic fluid causing rickettsial fluorescence.

^b —, Less than 1:100.

^c Obtained from Y. Kobayashi.

RESULTS

Characterization of MAbs. Eight clones of hybridoma were established (Table 1). Five MAbs (KP10, GM165, KT410, KAPMA, GILMA) reacted with the homologous strain in the IFA test with a titer ranging from 1:6,400 to 1:25,600 and with the heterologous strains with a titer of less than 1:100. So these MAbs were considered to be serotype-specific MAbs. One MAb (KP5) reacted with two of the prototypes (Karp, Kato) and one isolated strain (B92). One MAb (KATMA) reacted with one of the prototype strains (Kato) and an isolated strain (B92). KI4, which was obtained from a mouse immunized with B92, only reacted with B92. Therefore, this MAb was considered to be specific to Korean isolates.

Antigenic analysis of isolated strains. The antigenicity of *R. tsutsugamushi* was analyzed by an indirect IFA test with the eight MAbs and mouse polyclonal antibodies. Most of the isolated strains could be classified into one of the four groups according to their reaction patterns to these MAbs (Table 2). Some of the isolates showed an antigenicity pattern that was a mixture of those of two different strains.

Nine strains belonging to group I reacted with only Gilliam-specific MAbs (GM165, GILMA). Seven strains of group II reacted with two Karp-specific MAbs (KP10, KAPMA) and KP5. Six strains of group III reacted with one of the Karp-specific MAbs (KP10) and KP5. Eighty-eight strains of group IV reacted with KP5, KATMA, and KI4, which is specific for B92.

The reaction of the mouse hyperimmune sera with the prototype and isolated strains showed some cross-reaction, but each strain reacted with the highest titer against one specific anti-prototype serum (Table 3). The strains of group I reacted with the highest titer against the anti-Gilliam

TABLE 3. Reaction of *R. tsutsugamushi* strains with mouse hyperimmune sera

Rickettsial strain	Reaction with mouse hyperimmune serum:		
	Anti-Gilliam	Anti-Karp	Anti-Kato
Prototype			
Gilliam	320 ^a	40	40
Karp	40	320	40
Kato	40	40	320
Isolate group			
I	160–320	40–80	40–80
II	40–80	320	80
III	40	160–320	40–80
IV	40	160–320	40–160

^a Reciprocal of the highest dilution of mouse serum causing rickettsial fluorescence.

serum. The other strains of groups II, III, and IV reacted with the highest titer against the anti-Karp serum. No strain reacted with the highest titer against the anti-Kato serum.

These results indicated that the serotype of the group I strains is Gilliam and that of groups II and III is Karp. Group IV strains seem to be a new serotype that is closely related to Karp.

Two isolates reacted with KP10, KAPMA, KP5, GILMA, and GM165, and one isolate reacted with KP10, KP5, GILMA, and GM165. Thus the former seems to be the mixture of groups I and II, and the latter seems to be the mixture of groups I and III.

Geographical distribution of serotype. The prevalent serotype of *R. tsutsugamushi* in Korea differed according to geographical area (Table 4). In the northern part of Korea, such as Kyonggi and Kangwon provinces, 10 Gilliam strains and 10 Karp strains were isolated, whereas only 1 new serotype strain was isolated. But in the middle and southern part, such as Chungbuk, Chungnam, Chonbuk, and Kyongnam provinces, 87 strains of the new serotype, 6 strains of Karp, and 2 strains of Gilliam were isolated (Fig. 1).

DISCUSSION

The antigenic heterogeneity of *R. tsutsugamushi* has been documented by analysis with fluorescent antibody, complement fixation, cross-neutralization, cross-vaccination, or toxin neutralization (1, 6, 10, 14, 15). However, these methods present many difficulties, because there is a high degree of cross-reaction between the strains (4). Moreover, in addition to the classical prototype strains (Karp, Gilliam, and Kato), many new isolates that are antigenically different from the above three strains have been reported in Thailand

TABLE 2. Reaction patterns of 113 *R. tsutsugamushi* isolates with MAbs in the IFA test

Reaction group	Reaction pattern with MAb:								No. of isolates	Serotype
	GM165	GILMA	KP10	KAPMA	KP5	KT410	KATMA	KI4		
I	+	+	— ^b	—	—	—	—	—	9	Gilliam
II	—	—	+	+	+	—	—	—	7	Karp
III	—	—	+	—	+	—	—	—	6	Karp
IV	—	—	—	—	+	—	+	+	88	New serotype
I and II	+	+	+	+	+	—	—	—	2	Gilliam and Karp
I and III	+	+	+	—	+	—	—	—	1	Gilliam and Karp

^a +, Isolates could be stained in the IFA test with MAbs diluted more than 400-fold.

^b —, Isolates could not be stained in the IFA test with MAbs diluted 100-fold.

TABLE 4. Geographical distribution of serotypes

Province	No. of isolates			
	Gilliam	Karp	New serotype	Gilliam and Karp
Kyonggi	6	7	1	3
Kangwon	1	0	0	0
Chungbuk	0	4	1	0
Chungnam	2	2	62	0
Chonbuk	0	0	13	0
Kyongnam	0	0	11	0

and Japan (5, 16, 18). So it became more difficult to analyze such diverse antigenicity by conventional methods only. Recent studies suggest that MABs should be very useful reagents for classifying such diverse isolates of *R. tsutsugamushi* (4, 7).

We tried to identify the serotype of the *R. tsutsugamushi* isolates by an IFA test. Initially we used polyclonal antibodies from mice infected with three prototype strains. However, it was not possible to determine the serotype of many isolates because of serological cross-reaction (8). Therefore, we used MABs for the serological classification of the isolates.

Based on the reactions of MABs to group IV isolates (Table 2), the antigenic relationship between Karp, Kato and isolates can be presented as a schematic model (Fig. 2).

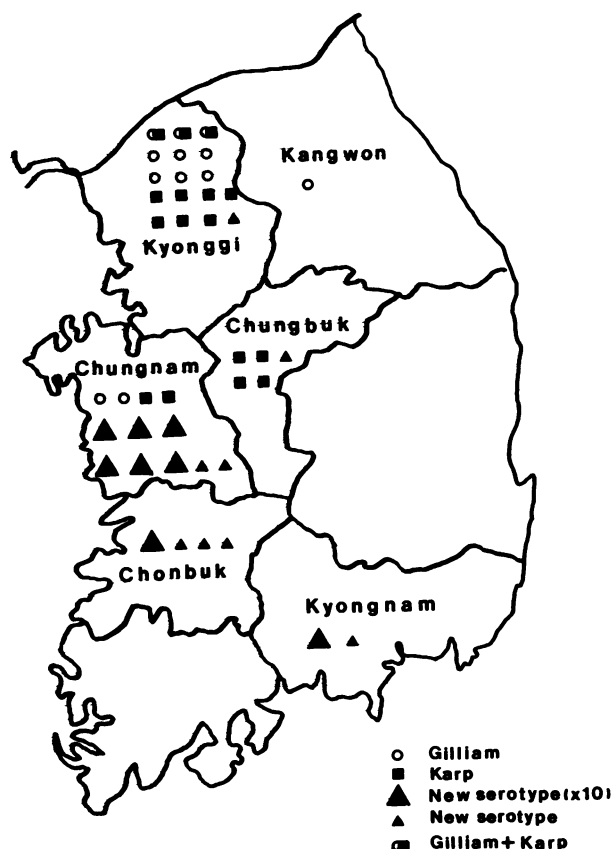


FIG. 1. Distribution of the serotypes of *R. tsutsugamushi* isolated in Korea.

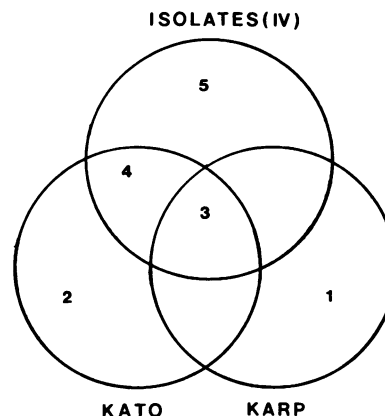


FIG. 2. Schematic presentation of the antigenic relationship among strains of *R. tsutsugamushi* (Karp, Kato, and isolated strains of group IV). Each circle represents the total number of antigens of each serotype strain. The numbers indicate the epitopes with which MABs (see the text) reacted.

Three prototype strains share common antigens. Karp and Kato strains also have common antigens (Fig. 2). Two Karp-specific MABs (KAPMA, KP10) reacted with epitopes on area 1. A Kato-specific MAB (KT410) reacted with epitopes on area 2, and 1 MAB (KATMA) reacted with epitopes on area 4. One MAB (KP5) reacted with epitopes on area 3, which is common for Karp, Kato, and B92. One MAB (KI4) reacted with epitopes on area 5, which is specific for group IV isolates. So these strains have common epitopes with Karp, Kato, and Gilliam strains (Table 3), but they also have their own unique epitopes.

However, in the course of this study, we also found that antigenic classification by MABs or polyclonal antibodies only can cause gross errors. The isolates of group I reacted with two Gilliam-specific MABs and can also be classified as Gilliam by polyclonal antibodies (Table 2). Isolates of groups II and III were classified as Karp by both the polyclonal antibodies and MABs. But isolates of group IV can be classified as Karp by polyclonal antibodies, but they reacted with neither of the two Karp-specific MABs (KP10, KAPMA). This conflicting result shows that isolates of group IV can be classified as new serotypes that are closely related to the Karp serotype.

In this study, we also found three cases of infection by multiple reactive strains. Such findings are very common among the isolates from Pakistan (13), the Philippines (11), and Thailand (12) but not common in Japanese isolates. Since the reactions of our MABs are highly specific, we can clearly confirm that these cases actually contain a mixture of two different serotypes.

The distribution of prevalent serotypes of the isolates from northern Korea is different from those of isolates from the middle and southern parts, and new serotype strains were isolated mainly from the middle and southern parts. Shirai and Wisseman (13) reported that no unique correlation was found between serotypes and local factors, and therefore a geographically specific diagnostic and prophylactic requirement would not be a serious problem. But our findings suggested that local strains of *R. tsutsugamushi* should be used for diagnostic and prophylactic purposes and that there might be different epidemiological characteristics, such as vector species, according to the locality.

In recent years, several investigators have reported new isolates that are antigenically different from the three proto-

type strains, so it is highly desirable to study the antigenic relationships among the new serotypes from various areas. Thus, we are trying to produce MABs that are reactive to the strains from Thailand (TA763, TA716, TA686, TH1817, TA678) and to do further comparative analysis with these strains.

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